

The Human Immunodeficiency Virus Type 1 Vif Protein Reduces Intracellular Expression and Inhibits Packaging of APOBEC3G (CEM15), a Cellular Inhibitor of Virus Infectivity

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Received 13 May 2003/Accepted 29 July 2003

Replication of human immunodeficiency virus type 1 (HIV-1) in most primary cells and some immortalized T-cell lines depends on the activity of the viral infectivity factor (Vif). Vif has the ability to counteract a cellular inhibitor, recently identified as CEM15, that blocks infectivity of Vif-defective HIV-1 variants. CEM15 is identical to APOBEC3G and belongs to a family of proteins involved in RNA and DNA deamination. We cloned APOBEC3G from a human kidney cDNA library and confirmed that the protein acts as a potent inhibitor of HIV replication and is sensitive to the activity of Vif. We found that wild-type Vif inhibits packaging of APOBEC3G into virus particles in a dose-dependent manner. In contrast, biologically inactive variants carrying in-frame deletions in various regions of Vif or mutation of two highly conserved cysteine residues did not inhibit packaging of APOBEC3G. Interestingly, expression of APOBEC3G in the presence of wild-type Vif not only affected viral packaging but also reduced its intracellular expression level. This effect was not seen in the presence of biologically inactive Vif variants. Pulse-chase analyses did not reveal a significant difference in the stability of APOBEC3G in the presence or absence of Vif. However, in the presence of Vif, the rate of synthesis of APOBEC3G was slightly reduced. The reduction of intracellular APOBEC3G in the presence of Vif does not fully account for the Vif-induced reduction of virus-associated APOBEC3G, suggesting that Vif may function at several levels to prevent packaging of APOBEC3G into virus particles.

The human immunodeficiency virus type 1 (HIV-1) accessory protein Vif plays an important role in regulating virus infectivity (10, 44). The lack of a functional Vif protein results in the production of virions with reduced or abolished infectivity (10, 23, 44). Despite this critical role of Vif in regulating virus infectivity, its mechanism of action has thus far remained obscure. Vif-deficient viruses can attach to and penetrate host cells but are blocked at a postpenetration step early in the infection cycle (2, 7, 8, 33, 40, 45). Yet comparison of virion morphology or protein composition between wild-type and Vif-defective virions has produced conflicting results (4, 6, 12, 14, 18, 31, 37). Several reports have suggested that Vif affects the stability of the viral nucleoprotein complex (18, 32, 40). In particular, NC and reverse transcriptase were found to be less stably associated with viral cores in the absence of Vif, suggesting a role for Vif in the proper assembly of the nucleoprotein complex (32). This is consistent with our recent observation that Vif is specifically packaged into HIV-1 particles (21).

There is increasing evidence that packaging of Vif into virus particles is functionally relevant. For example, Vif packaging is specific and is mediated through an interaction with viral genomic RNA (21). In addition, virus-associated Vif interacts with Gag and/or Gag-Pol precursor molecules (H. Akari and K. Strebel, unpublished data) and is stably associated with the viral nucleoprotein complex. Finally, virus-associated Vif is proteolytically cleaved by the viral protease at a conserved

sequence located near the C terminus of the protein (22). Interestingly, mutations at or near the processing site that affect Vif processing also were found to affect Vif function, while mutations that did not affect Vif processing also did not affect Vif function (22).

The effect of Vif on virus infectivity is producer cell dependent and can vary by several orders of magnitude (for review, see references 5 and 43). Virus replication in nonpermissive cell types such as primary T cells and macrophages as well as a small number of T-cell lines, including H9, is strictly dependent on Vif. In contrast, Vif-defective viruses can efficiently replicate in permissive hosts such as Jurkat cells. Results from heterokaryon analyses which involved the fusion of restrictive with permissive cell types suggested the presence of an inhibitory factor in restrictive cells (27, 39). Recent work by Sheehy et al. identified a cellular factor, CEM15, which was expressed in cell types that are restrictive for the replication of Vif-defective viruses but was not expressed in permissive cell types (36). Expression of CEM15 in permissive cell types imposed a restrictive phenotype on these cells, providing intriguing evidence that CEM15 is indeed a cellular inhibitor whose activity must be overcome by Vif for HIV replication to proceed (36). Interestingly, CEM15, like Vif, is packaged into virions (36). Sequence comparison revealed a significant homology of CEM15 with APOBEC-1, a member of the APOBEC family of RNA editing enzymes (36). In fact, CEM15 is identical to APOBEC3G, for which cytidine deaminase activity was demonstrated in vitro (16). Most recently, a series of papers demonstrated that APOBEC3G induces hypermutation of viral

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cDNA in the absence of Vif (15, 24, 29, 47). A possible mechanism for Vif function therefore involves inactivation of CEM15.

The goal of the present study was to characterize the effect of APOBEC3G on HIV infectivity in the presence or absence of Vif and to gain insights into the mechanism of APOBEC3G neutralization by Vif. For this purpose, we cloned APOBEC3G from a human kidney cDNA library. Sequence analysis revealed that the clone obtained encoded a gene that was identical to APOBEC3G (GenBank no. NM_021822) and MDS019 (GenBank no. AF182420) except for two amino acid residues ($S_{162}N$ and $D_{370}Y$). Expression of APOBEC3G in HeLa cells confirmed that the protein was biologically active and severely inhibited virus infectivity in the absence of Vif. Analysis of virus preparations revealed that packaging of APOBEC3G was inhibited by wild-type Vif but not by a series of biologically inactive Vif variants. The inhibition of APOBEC3G packaging by wild-type Vif was dose dependent. Interestingly, increasing levels of APOBEC3G did not adversely affect Vif packaging. Analysis of the intracellular APOBEC3G expression levels suggests that wild-type Vif but not biologically inactive Vif variants decrease steady-state levels of APOBEC3G. Finally, kinetic analyses suggest that Vif does not increase turnover of cell-associated APOBEC3G but instead reduces its rate of synthesis.

MATERIALS AND METHODS

Plasmids. The full-length molecular clone pNL4-3 (1) was used for the production of wild-type infectious virus. For transient expression of Vif, the subgenomic expression vector pNL-A1 (44) was employed. This plasmid expresses all HIV-1 proteins except for *gag* and *pol* products. A Vif-defective variant, pNL-A1vif(-), carrying an *NdeI-Pf*MI deletion in the *vif* gene (20) was used as a negative control and as filler DNA in some of the experiments. Plasmids pcDNA-APO3G and pHIV-APO3G are vectors for the expression of APOBEC3G under the control of the cytomegalovirus immediate-early promoter and the HIV promoter, respectively. In-frame deletions in *vif* were introduced into pNL-A1 by PCR-based mutagenesis. Similarly, mutation of Cys_{114} and Cys_{133} to Ser in Vif was accomplished by PCR-based mutagenesis of pNL-A1. All variants were verified by sequence analysis.

Antisera. Serum from an HIV-positive patient (AIDS patient serum [APS]) was used to detect HIV-1-specific proteins. The serum does not recognize Vif or Nef and only poorly reacts with gp120 in immunoblot assays. A monoclonal antibody against Vif (MAb 319) was used for all immunoblot analyses and was obtained from Michael Malim through the NIH AIDS Research and Reference Reagent Program (11, 12, 38, 42). For immunocytochemical analyses, our Vif-specific polyclonal antibody (Vif93) was employed. APOBEC3G was identified using an horseradish peroxidase (HRP)-conjugated anti-His (C-terminal) monoclonal antibody (Invitrogen Corp., Carlsbad, Calif.) or a polyclonal rabbit serum against recombinant APOBEC3G. Immunoprecipitation of APOBEC3G from transfected HeLa cells was done with a Myc epitope tag-specific rabbit polyclonal antibody (Sigma-Aldrich, Inc., St. Louis, Mo.). A monoclonal antibody against α -tubulin was purchased from Sigma-Aldrich.

Tissue culture and transfections. HeLa cells were propagated in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (FBS). LuSIV cells are derived from CEMx174 cells and contain a luciferase indicator gene under the control of the SIVmac239 long terminal repeat (LTR) (34). These cells were obtained through the NIH AIDS Research and Reference Reagent Program and were maintained in complete RPMI 1640 medium supplemented with 10% FBS and hygromycin B (300 μ g/ml).

For transfection of HeLa cells, cells were grown in 25-cm² flasks to about 80% confluency. Cells were transfected using Lipofectamine PLUS (Invitrogen Corp.) following the manufacturer's recommendations. A total of 5 to 6 μ g of plasmid DNA per 25-cm² flask was used. Cells were harvested at 24 to 48 h posttransfection.

Preparation of virus stocks. Virus stocks were prepared by transfecting HeLa cells with appropriate plasmid DNAs. Virus-containing supernatants were harvested 24 to 48 h after transfection. Cellular debris was removed by centrifugation (3 min, 3,000 \times g), and clarified supernatants were filtered (0.45- μ m-pore-size filter) to remove residual cellular contaminants. HeLa cell-derived virus

preparations generally exhibit low levels of microvesicle contamination. Virus preparations were therefore purified by a simplified method by centrifugation through 20% sucrose. However, virus association of APOBEC3G was verified in pilot experiments by treating viruses with subtilisin followed by linear sucrose gradient centrifugation and immunoblotting with APOBEC3G-specific antiserum (data not shown).

Immunoblotting. For immunoblot analysis of intracellular proteins, whole-cell lysates were prepared as follows. Cells were washed once with phosphate-buffered saline, suspended in phosphate-buffered saline (400 μ l/10⁷ cells), and mixed with an equal volume of sample buffer (4% sodium dodecyl sulfate [SDS], 125 mM Tris-HCl [pH 6.8], 10% 2-mercaptoethanol, 10% glycerol, and 0.002% bromophenol blue). Proteins were solubilized by boiling for 10 to 15 min at 95°C, with occasional mixing of the samples on a vortex mixer to shear chromosomal DNA. Residual insoluble material was removed by centrifugation (2 min, 15,000 rpm in an Eppendorf Minifuge). Cell lysates were subjected to SDS-polyacrylamide gel electrophoresis; proteins were transferred to polyvinylidene difluoride membranes and reacted with appropriate antibodies as described in the text. Membranes were then incubated with horseradish peroxidase-conjugated secondary antibodies (Amersham Biosciences, Piscataway, N.J.) and visualized by enhanced chemiluminescence (ECL; Amersham Biosciences). Quantitation of protein levels was done by densitometric scanning of appropriate exposures to BioMax Light film (Eastman Kodak, Rochester, N.Y.). Multiple exposures were collected to ensure that all protein bands on a blot were within the linear range of the film. Data analysis was done using Image Gauge, version 3.45, software (Fuji Photofilm LTD).

Total RNA isolation and Northern blot analysis. Total RNA was prepared by using RNeasy Mini kits (Qiagen, Valencia, Calif.). RNA samples were electrophoresed on denatured 1.2% agarose gels and capillary blotted onto a nylon membrane (Schleicher & Schuell, Inc., Keene, N.H.) by using a Turbo blotter (Schleicher & Schuell, Inc.). After UV cross-linking, the membranes were pre-hybridized with 10 ml of QuickHyb hybridization solution (Stratagene, La Jolla, Calif.) for 1 h at 68°C and then incubated with probes for 5 h at 68°C. Probes were labeled with [³²P]deoxy-CTP by using the random primer-based Ladder-man labeling kit (PanVera, Madison, Wis.). Probes were mixed with 100 μ l of sonicated salmon sperm DNA (10 mg/ml; Stratagene), heated at 94°C for 5 min, and then chilled on ice. Labeled probes (10⁷ cpm) in 10 ml of hybridization buffer were used for each experiment. After hybridization, membranes were washed twice with washing buffer (2 \times SSPE [1 \times SSPE is 0.18 M NaCl, 10 mM NaH₂PO₄, and 1 mM EDTA {pH 7.7}], 0.1% SDS) for 15 min at room temperature, followed by one wash with 0.2 \times SSPE-0.1% SDS for 15 min at 60°C. For reprobing, membranes were stripped by incubation in 1% SDS in H₂O for 15 min at 100°C. To detect APOBEC3G mRNA, a 1-kb cDNA fragment derived from pcDNA-APO3G was used. Actin mRNA was identified by using a 500-bp beta-actin cDNA fragment.

Metabolic labeling and immunoprecipitation. For pulse-chase experiments, transfected HeLa cells were scraped off the flasks at 18 to 20 h posttransfection, washed, and then starved for 10 min in methionine-free RPMI 1640. Cells were pulse-labeled for 30 min in 200 μ l of methionine-free RPMI 1640 containing 400 μ Ci of [³⁵S]methionine (ICN Biomedical Inc., Aurora, Ohio) and chased for 0, 0.5, 1, or 2 h in complete RPMI 1640-10% FBS. Cells were lysed in a buffer containing 50 mM Tris-hydrochloride (pH 8.0), 5 mM EDTA, 100 mM NaCl, 0.5% (wt/vol) 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS), and 0.2% (wt/vol) deoxycholate (3). Cell lysates were precleared by incubation at 4°C for 1 h with protein A-Sepharose CL4B (Sigma-Aldrich, Inc.). Cleared cell lysates were used for immunoprecipitation of APOBEC3G using a Myc-specific polyclonal rabbit antibody (Sigma-Aldrich, Inc.). Immunoprecipitated proteins were solubilized by boiling in sample buffer containing 2% SDS, 1% β -mercaptoethanol, 1% glycerol, and 65 mM Tris-hydrochloride (pH 6.8) and were separated on 11% polyacrylamide-SDS gels. Gels were soaked in 1 M sodium salicylic acid for 30 min and dried. Radioactive bands were visualized by fluorography. Quantitation of the radioactivity of the respective bands was performed with a Fuji BAS 2000 Bio-Image Analyzer.

Infectivity assay. LuSIV cells (5×10^5) were infected in a 24-well plate with 200 to 400 μ l of unconcentrated virus supernatant. Cells were incubated for 24 h at 37°C. Cells were then harvested and lysed in 150 μ l of Promega 1 \times reporter lysis buffer (Promega Corp., Madison, Wis.). To determine the luciferase activity in the lysates, 50 μ l of each lysate was combined with luciferase substrate (Promega Corp.) by automatic injection, and light emission was measured for 10 s at room temperature in a luminometer (Optocomp II; MGM Instruments, Hamden, Conn.).

RESULTS

Cloning and characterization of MDS019/APOBEC3G. Database searches revealed that CEM15 was identical to MDS019, a phorbolin-like protein of unknown function (36). To verify that CEM15 and MDS019 were structurally and functionally equivalent and to independently verify the activity of CEM15 on HIV replication, we cloned MDS019 from a human kidney cDNA library (ResGen; Invitrogen Corp.) by using two sets of PCR primers whose sequences were designed based on the published sequence for MDS019 (GenBank no. AF182420). For the primary amplification, primers 5' GGGA CTAGCCGGCCAAGGATG and 3' CCTTAGAGACTGAG GCCATCCTTC were used to amplify MDS019 by standard PCR. These primers were designed for maximum fit with the template DNA and did not contain cloning sites. The primary PCR product was subsequently reamplified by using oligonucleotides 5' CATAGAATTCAAGGATGAAGCCTCACTT CAG and 3' GTATAAGCTTGTTCCTGATTCTGGAG, containing *Eco*RI and *Hind*III cloning sites (underlined), respectively, and cloned into pcDNA3.1(-)/MycHis (Invitrogen Corp.). This strategy removed the stop codon from the MDS019 open reading frame and generated an in-frame fusion with the C-terminal MycHis epitope encoded by the cloning vector. Sequence analysis of the resulting construct, pcDNA-APO3G:MycHis (also referred to in the text as pcDNA-APO3G), verified that the amplified gene was identical to MDS019 and APOBEC3G (NM_021822; *Homo sapiens* apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3G) (19), except for two nucleotide changes that resulted in two amino acid changes (S₁₆₂N and D₃₇₀Y). Unlike CEM15 or MDS019, for which no functional data are available, APOBEC3G was found to have deaminase activity in vitro (16). Because of the lack of functional data for MDS019 and the overall similarity of the protein with other members of the APOBEC family (16, 36), we decided to use the term APOBEC3G instead of CEM15 or MDS019 when referring to our protein. To render expression of APOBEC3G dependent on the coexpression of HIV proteins, the APOBEC3G-MycHis chimera was PCR amplified and cloned between the HIV-1 LTRs by using the *Bss*HII and *Xho*I sites in pNL4-3 (1). The APOBEC3G expression vector encodes a protein of 410 amino acids (385 residues of APOBEC3G and 25 residues corresponding to the MycHis epitope tag) with a predicted molecular mass of 49.3 kDa. Indeed, immunoblot analysis of transiently transfected HeLa cells using a His-specific antibody revealed a protein of approximately 45 kDa (not shown).

Expression of APOBEC3G blocks HIV-1 infectivity. To determine whether our APOBEC3G clone was functionally equivalent to CEM15, wild-type or Vif-defective virus stocks were produced in HeLa cells in the presence or absence of increasing amounts of APOBEC3G. The infectivity of the resulting viruses was determined by infection of LuSIV indicator cells (34). As shown in Fig. 1A, transfection into HeLa cells of increasing amounts of pcDNA-APO3G together with pNL4-3 (Vif +) or pNL4-3Vif(-) (Vif -) resulted in the increased intracellular expression as well as packaging of APOBEC3G into cell-free virions (Fig. 1A, panel 3G). Expression of APOBEC3G did not affect expression of viral proteins, as indicated by the constant level of cell-associated capsid protein

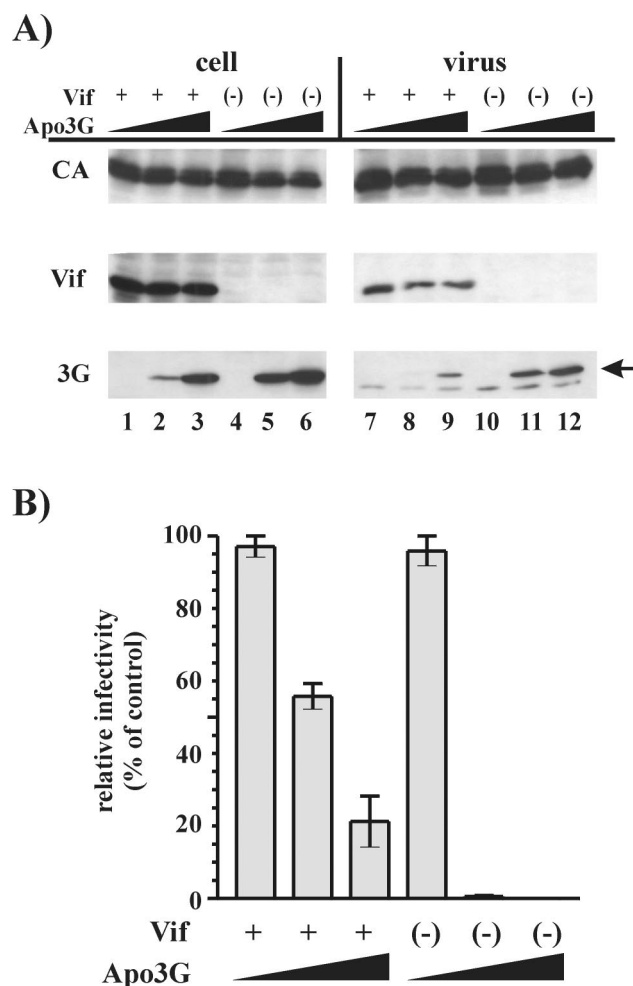


FIG. 1. APOBEC3G inhibits HIV-1 infectivity. HeLa cells were transfected with 2.5 μ g of pNL4-3 (Vif +) or pNL4-3Vif(-) (Vif -) together with 0, 1, or 2.5 μ g of pcDNA-APO3G. All samples were adjusted to a total of 5 μ g of DNA per transfection with empty vector DNA (pcDNA3.1MycHis). Cells and virus-containing supernatants were harvested 24 h later. A fraction of the virus (80%) was pelleted through a 20% sucrose cushion. The remaining virus was used for reverse transcription assay and infectivity analyses. (A) Whole-cell lysates (5% of total) and viral pellets (25% of total) were separated by SDS-13% polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride membranes, and reacted with a His-specific, HRP-conjugated monoclonal antibody (Invitrogen). Proteins were visualized by ECL (3G). The position of APOBEC3G is marked by an arrow. A nonspecific background band with slightly faster mobility was detected by the His-specific antiserum in this and some of the other experiments. The same blot was subsequently reblotted first with a Vif-specific monoclonal antibody (Vif), followed by an HIV-positive human serum (CA). (B) Infectivities of the viruses produced for panel A were determined by infecting LuSIV cells with equal amounts of virus as described in Materials and Methods. Infectivities of samples in the absence of APOBEC3G were defined as 100% for wild-type and Vif-defective viruses.

(Fig. 1A, panel CA, lanes 1 to 6). Furthermore, APOBEC3G expression had no effect on the release of viral proteins (Fig. 1A, panel CA, lanes 7 to 12) and did not appear to affect packaging of Vif (Fig. 1A, panel Vif, lanes 7 to 9). However, expression of APOBEC3G had a dramatic impact on the infectivity of the viruses produced in the absence of Vif, as

indicated by the >200-fold reduction of virus-induced luciferase activity (Fig. 1B, Vif $-$). Interestingly, APOBEC3G expression also reduced the infectivity of Vif-expressing wild-type virus. However, the inhibitory effect was minor when compared to the effect seen in the absence of Vif (Fig. 1B, Vif $+$). These data are consistent with those reported by Sheehy et al. (36) and confirm that pcDNA-APO3G encodes a biologically active protein capable of inhibiting the infectivity of vif-defective HIV virions.

Vif inhibits packaging of APOBEC3G. It is interesting that both Vif and APOBEC3G proteins are packaged into virions. To investigate the possible correlation of APOBEC3G packaging and its inhibitory effect on virus infectivity, we analyzed the effect of Vif on APOBEC3G packaging (Fig. 2). For this purpose, HeLa cells were transfected with increasing amounts of the Vif expression vector pNL-A1 in the presence of constant amounts of Vif-defective pNL43Vif($-$) proviral DNA and pcDNA-APO3G (Fig. 2A, lanes 4 to 6 and 10 to 12). At the same time, we wanted to investigate possible effects of APOBEC3G on Vif packaging. This was accomplished by expressing physiological levels of Vif from the wild-type pNL4-3 proviral vector in the presence of increasing amounts of APOBEC3G (Fig. 2A, lanes 1 to 3 and 7 to 9). Cell lysates and concentrated virus fractions were analyzed by immunoblotting with an HIV-positive patient serum recognizing capsid (CA), a histidine-specific antibody recognizing APOBEC3G (3G), or a Vif-specific monoclonal antibody (Vif). All samples contained similar amounts of intracellular and virus-associated Gag proteins (Fig. 2A, panel CA), attesting to comparable transfection efficiencies and to the absence of an inhibitory effect of APOBEC3G on the synthesis of viral proteins. Consistent with the results from Fig. 1, expression of increasing levels of APOBEC3G (panel 3G, lanes 1 to 3) in the presence of constant amounts of Vif (panel Vif, lanes 1 to 3) resulted in increased packaging of APOBEC3G (panel 3G, lanes 7 to 9) but did not affect Vif packaging (panel Vif, lanes 7 to 9). In contrast, expression of increasing amounts of Vif (panel Vif, lanes 4 to 6) in the presence of constant amounts of APOBEC3G (panel 3G, lanes 4 to 6) resulted in decreasing amounts of virus-associated APOBEC3G (panel 3G, lanes 10 to 12) paralleled by increased packaging of Vif (panel Vif, lanes 10 to 12). The relationship between Vif expression and APOBEC3G packaging was quantified by calculating the fraction of virus-associated APOBEC3G from the total intra- and extracellular protein (Fig. 2B). It is interesting that at constant levels of Vif (Fig. 2B, columns 1 to 3) and increasing levels of APOBEC3G, an increased proportion of total available APOBEC3G was packaged (10 and 21%, respectively). This suggests that under such conditions, Vif is overwhelmed by the high levels of APOBEC3G and can only minimally control APOBEC3G packaging. This view is supported by the notion that expression of similar levels of APOBEC3G in the complete absence of Vif (column 4) resulted in an only minor additional increase in APOBEC3G packaging efficiency (26%). Importantly, however, at constant levels of APOBEC3G (columns 4 to 6), increased expression of Vif progressively inhibited the proportion of virus-associated APOBEC3G (from 26% to less than 10%). These results demonstrate that Vif inhibits packaging of APOBEC3G in a dose-dependent manner.

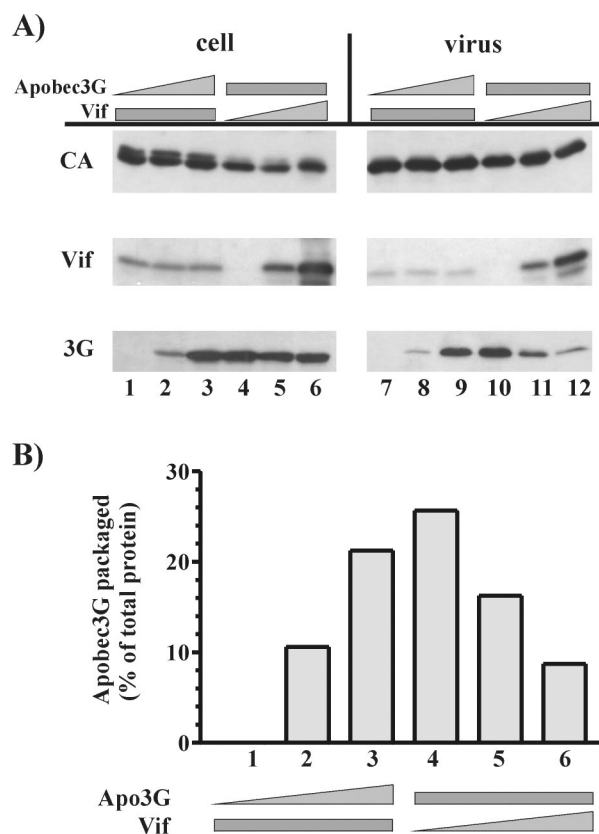


FIG. 2. Vif inhibits packaging of APOBEC3G. (A) To determine the effects of APOBEC3G on Vif packaging, HeLa cells were transfected with 2.5 μ g of pNL4-3 (expressing constant amounts of Vif) and 0, 0.5, or 2.5 μ g of pcDNA-APO3G. Total DNA was adjusted in all samples to 5 μ g with empty vector DNA. Samples were subjected to immunoblot analysis as described in the text. Lanes 1 to 3 and 7 to 9 represent cell and viral lysates, respectively. To determine the effect of Vif on APOBEC3G packaging, HeLa cells were transfected with 1.5 μ g of pcDNA-APO3G together with 2.5 μ g of the Vif-defective pNL4-3Vif($-$) and 0, 0.5, or 2 μ g of the Vif expression vector pNL-A1. Total DNA was adjusted to 6 μ g with Vif-defective pNL-A1Vif($-$) DNA. Lanes 4 to 6 and 10 to 12 represent cell and viral lysates, respectively. Capsid proteins were identified with an HIV-positive patient serum (CA). APOBEC3G was identified by using an HRP-conjugated anti-His monoclonal antibody (3G), and Vif was identified by using a Vif monoclonal antibody. (B) Packaging of APOBEC3G was quantified by densitometric scanning of the gels shown in panel A. Appropriate exposures were chosen to ensure that signal intensities were within the linear range of the X-ray film. The percentage of virus-associated protein relative to the total intracellular plus extracellular protein was calculated. Values were corrected for differences in the amount of capsid protein and loading volumes.

Inhibition of APOBEC3G packaging requires biologically active Vif. Vif and APOBEC3G are both RNA binding proteins (9, 19, 21, 46). It is therefore possible that packaging of Vif and APOBEC3G requires interaction with a common motif on the viral genomic RNA. Assuming that Vif has a higher binding affinity to such a putative packaging signal, Vif could efficiently compete for packaging of APOBEC3G in a manner consistent with the results observed in Fig. 2. In such a scenario, competitive inhibition of APOBEC3G packaging by Vif would not necessarily require biologically active Vif but could be accomplished by any Vif variant that is efficiently packaged.

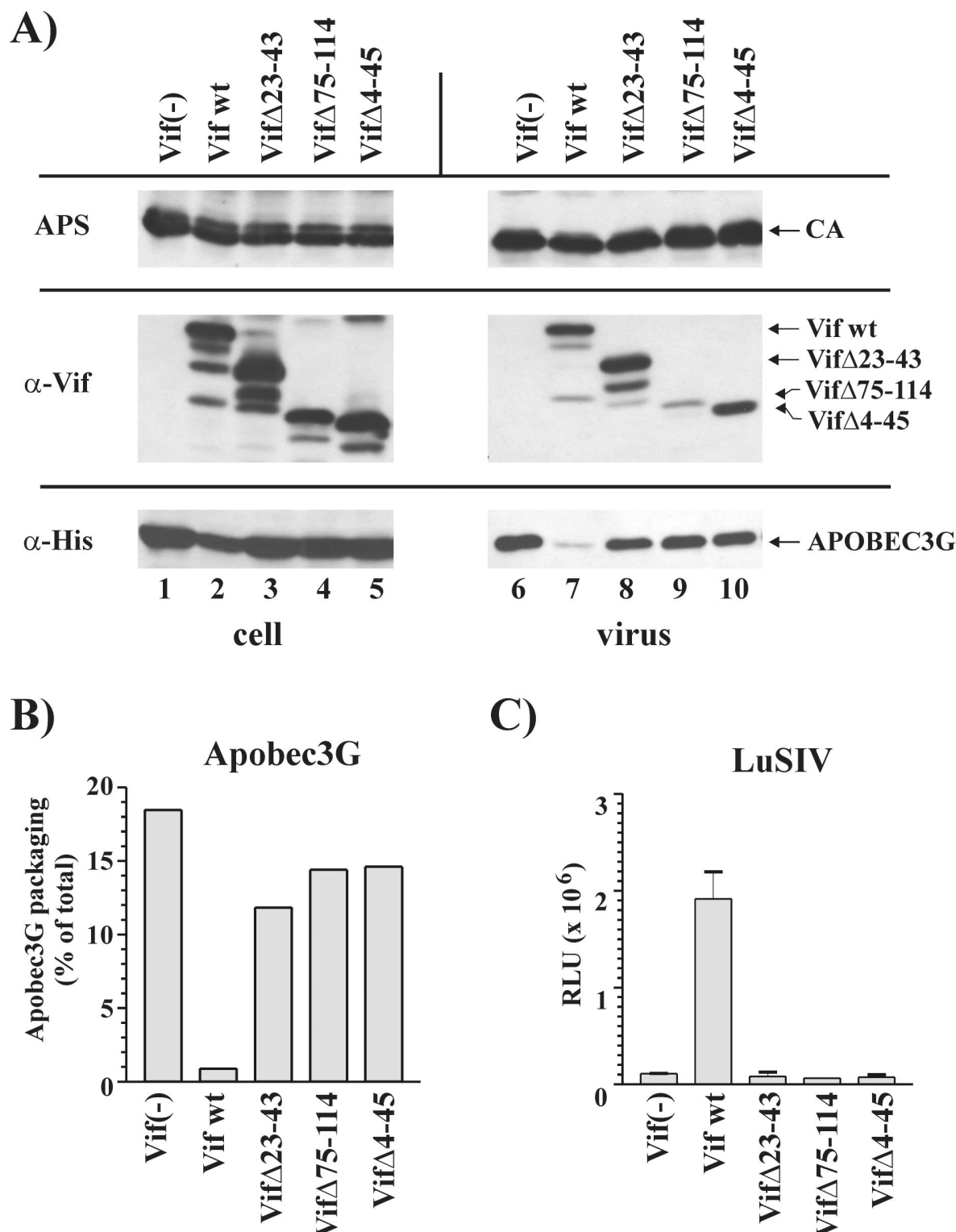


FIG. 3. Inhibition of APOBEC3G packaging requires biologically active Vif. (A) HeLa cells were transfected with 2.5 μ g of pNL4-3Vif(-), 1.5 μ g of pcDNA-APO3G, and 1.5 μ g of individual pNL-A1 variants. Lanes 1 and 6, Vif-deficient control; lanes 2 and 7, wild-type Vif. The other samples express Vif proteins carrying various in-frame deletions as indicated. Whole-cell lysates and virus fractions were prepared as described in the text and subjected to immunoblotting using an HIV-positive patient serum (APS) to recognize capsid proteins (CA), a Vif-specific monoclonal antibody (α -Vif), or a His-specific monoclonal antibody to detect APOBEC3G (α -His). The position of Vif variants is indicated on the right. (B) Packaging of APOBEC3G was quantitated by calculating the amounts of virus-associated proteins relative to the total intra- and extracellular proteins as described for Fig. 2. (C) LuSIV indicator cells were infected with comparable amounts of virus as determined by reverse transcriptase activity. Cells were harvested 24 h after infection and luciferase activity was determined as described in Materials and Methods. RLU, relative light units.

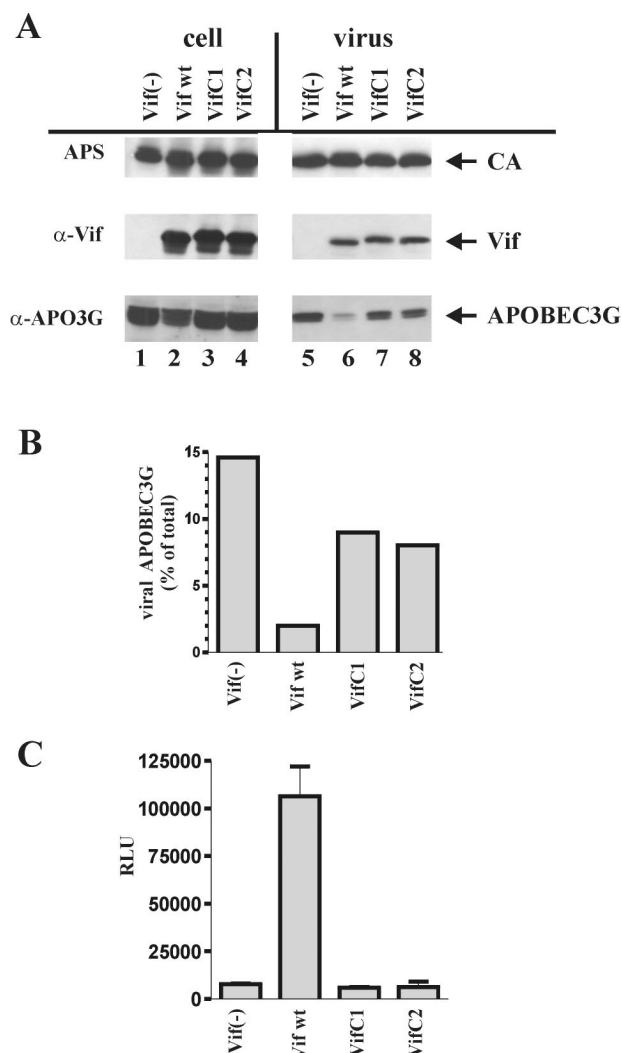


FIG. 4. Two conserved cysteine residues in Vif are required to inhibit packaging of APOBEC3G. (A) HeLa cells were transfected with 2 μ g of pNL4-3Vif(-), 1 μ g of pcDNA-APO3G, and 2 μ g of various pNL-A1 variants. Lanes 1 and 5, Vif-deficient control; lanes 2 and 6, wild-type Vif; lanes 3 and 7, VifC1 variants; lanes 4 and 8, VifC2 variants. Whole-cell lysates and virus fractions were prepared as described in the text and subjected to immunoblotting using an HIV-positive patient serum (APS) to recognize capsid proteins (CA), a Vif-specific monoclonal antibody (α -Vif), or an APOBEC3G-specific polyclonal antibody (α -APO3G). (B) Packaging of APOBEC3G was quantitated by calculating the amounts of virus-associated proteins relative to the total intra- and extracellular proteins as described for Fig. 3. (C) LuSIV indicator cells were infected with comparable amounts of virus as determined by RT activity. Cells were harvested 24 h after infection and luciferase activity was determined as described in Materials and Methods. RLU, relative light units.

To test this possibility, we analyzed a series of Vif variants carrying a variety of in-frame deletions (Fig. 3). To avoid potential problems with the replication capacity of our proviral construct, all Vif variants (including wild-type Vif) were expressed in *trans* from a separate plasmid. Thus, HeLa cells were transfected with a Vif-defective proviral construct [pNL43-Vif(-)] together with pcDNA-APO3G as well as the Vif expression vector pNL-A1 or one of its variants as indicated in Fig. 3A. As can be seen, all samples expressed similar amounts

of intracellular capsid protein (Fig. 3A, panel APS, lanes 1 to 5) and similar amounts of virus were produced in all cases as judged by the comparable levels of capsid protein (panel APS, lanes 6 to 10). All Vif variants were efficiently expressed intracellularly (panel α -Vif, lanes 2 to 5) and packaged with varying efficiencies (panel α -Vif, lanes 7 to 10). Intracellular expression levels of APOBEC3G were similar in all samples (panel α -His, lanes 1 to 5), except in the presence of wild-type Vif, for which the cell-associated level of APOBEC3G was slightly reduced (panel α -His, lane 2). Consistent with the results from Fig. 2, expression of wild-type Vif resulted in a severe inhibition of APOBEC3G packaging (compare panel α -His, lanes 6 and 7, and Fig. 3B). Surprisingly, none of the biologically inactive Vif variants tested was capable of blocking APOBEC3G packaging (panel α -His, lanes 8 to 10). Quantitation of the data shown in Fig. 3A confirmed that only wild-type Vif is capable of significantly inhibiting APOBEC3G packaging even when corrected for the somewhat reduced intracellular levels of APOBEC3G (Fig. 3B). The infectivities of the viruses produced in this experiment were determined by infection of LuSIV indicator cells as described for Fig. 1. As expected, only virus produced in the presence of wild-type Vif was capable of initiating a productive infection of the indicator cells (Fig. 3C). These results demonstrate that packaging of Vif alone is not sufficient to compete for APOBEC3G incorporation. Furthermore, these data point to a correlation between the inhibitory effect of APOBEC3G and its presence or absence in virions and suggest a virus-associated activity of APOBEC3G that interferes with the infectivity of virus particles.

The Vif mutants analyzed in Fig. 3 carry in-frame deletions of 21 to 42 residues. To further validate the conclusions drawn from those experiments, we employed mutants carrying more subtle changes in Vif. To do so, we analyzed Vif variants carrying mutations of two cysteine residues, Cys₁₁₄ and Cys₁₃₃, in HIV-1 Vif (Fig. 4). These two cysteine residues are highly conserved in Vif and have been shown to be crucial for its biological activity (13, 26, 35, 41). Mutations were created by PCR-based mutagenesis and expressed in the context of pNL-A1. pNL-A1-VifC1 and pNL-A1-VifC2 contain cysteine-to-serine changes at positions 114 and 133 of Vif, respectively. The effects of VifC1 and VifC2 on packaging of APOBEC3G were analyzed in HeLa cells as described for Fig. 3. As can be seen in Fig. 4A, both mutants were expressed and packaged into virions with efficiencies similar to that of wild-type Vif (Fig. 4A, panel α -Vif, compare lanes 3 and 4 to lane 2 and lanes 7 and 8 to lane 6). Also, the mutation of Vif cysteine residues had no measurable effect on expression or release of viral Gag proteins (Fig. 4A, panel APS). Quantitation of the packaging efficiency of APOBEC3G in Fig. 4B revealed that both cysteine mutants of Vif were significantly impaired in the ability to inhibit packaging of APOBEC3G. Consistent with previous reports, mutation of either cysteine residue in Vif resulted in a complete loss of infectivity in a single cycle infection assay (Fig. 4C). These results further support our conclusion that inhibition of APOBEC3G packaging requires biologically active Vif.

Vif reduces intracellular steady-state levels of APOBEC3G.

We noticed in some of our experiments (Fig. 2 and 3) that coexpression of wild-type Vif and APOBEC3G correlated with reduced levels of cell-associated APOBEC3G. To directly test

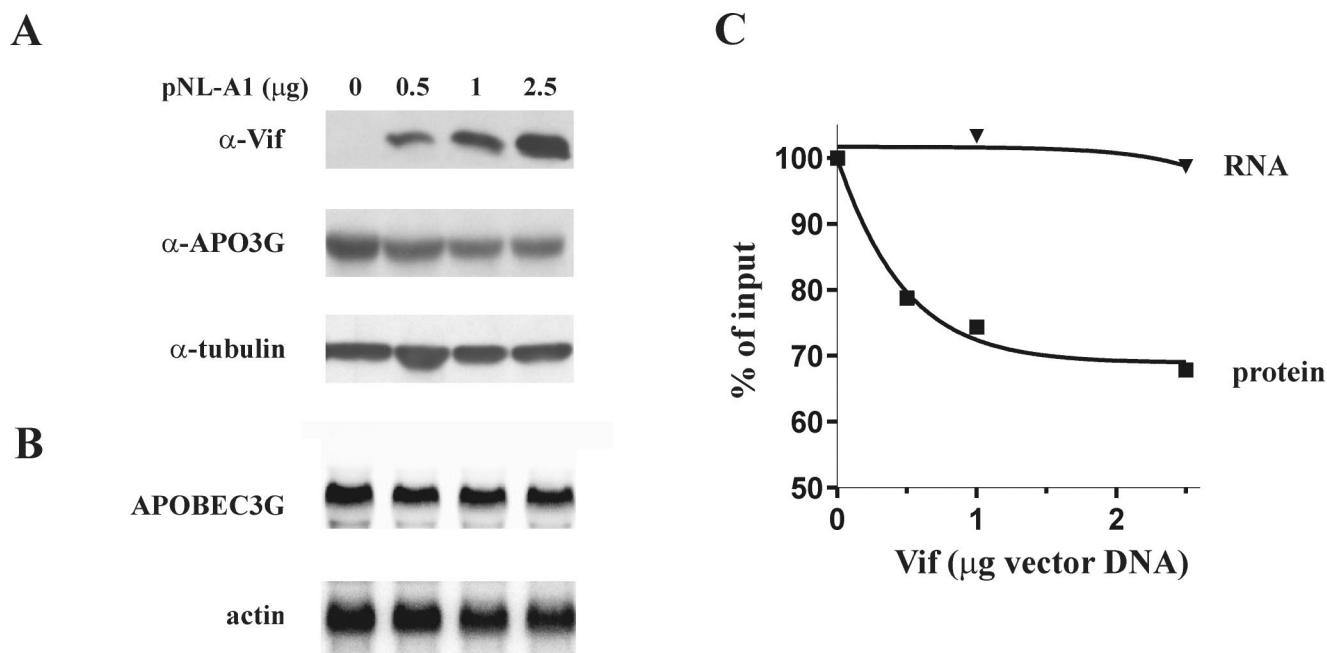


FIG. 5. Vif reduces intracellular levels of APOBEC3G. Duplicate flasks of HeLa cells (25 cm²) were each transfected with 2 μg of pHIV-APO3G and 0, 0.5, 1, or 2.5 μg of pNL-A1 plasmid DNA. All samples were adjusted to 5 μg of total DNA with pNL-A1Vif(−) DNA. Cells were harvested 24 h after transfection. Duplicate samples were pooled, and half of the cells were used to prepare whole-cell lysates for immunoblot analysis while the other half was used for preparation of total RNA for Northern blot analysis. (A) Cell lysates were subjected to immunoblotting using an APOBEC3G-specific polyclonal antiserum (α-APO3G) or a Vif-specific monoclonal antibody (α-Vif). To control for loading errors, the APOBEC3G blot was subsequently reblotted using an antibody against α-tubulin. (B) Total RNA was used for Northern blot analysis of APOBEC3G mRNA as described in Materials and Methods. For an internal control, an actin-specific probe was employed. (C) APOBEC3G-specific protein bands were quantified by densitometric scanning of the blot shown in panel A. APOBEC3G RNA signals shown in panel B were quantified using a Fuji phosphorimager. Protein signals were corrected for variations in the tubulin signal; RNA signals were normalized for actin. Results were calculated as percentages of the signals obtained in the absence of Vif (0) and plotted as a function of Vif expression.

the effect of Vif on intracellular expression of APOBEC3G, we compared the steady-state levels of APOBEC3G in the absence or presence of increasing amounts of Vif (Fig. 5). To rule out protein loss due to viral packaging, we performed the experiment in the absence of virus production. Also, to restrict APOBEC3G expression to Vif-expressing cells, we employed an HIV-1 LTR-driven expression vector, pHIV-APO3G. Efficient expression of APOBEC3G from this vector requires the transcriptional activator Tat, which is encoded by the Vif expression vector pNL-A1 (44). HeLa cells were transfected with a constant amount of pHIV-APO3G (2 μg) and increasing amounts of pNL-A1 (0, 0.5, 1, or 2.5 μg). All samples were adjusted to 5 μg of total DNA with the *vif*-defective variant, pNL-A1vif(−). Cells were harvested 24 h after transfection. Half of the cells were used for preparation of whole-cell lysates for immunoblotting (Fig. 5A); the second half was used to prepare total cellular RNA for use in Northern blotting (Fig. 5B).

Transfection of increasing amounts of Vif-expressing pNL-A1 plasmid DNA resulted in increasing amounts of Vif protein (Fig. 5A, panel α-Vif) and decreasing amounts of APOBEC3G protein (panel α-APO3G). Quantitation of APOBEC3G signals (Fig. 5C, protein) revealed a 20 to 30% decrease in APOBEC3G steady-state levels in Vif-expressing cells. Note that the reduction of APOBEC3G was not linearly dependent on Vif expression levels but reached a plateau with about 1 μg

of Vif expression vector. Increasing Vif expression beyond that point had only a marginal effect on APOBEC3G steady-state levels (Fig. 5C, protein), suggesting that saturating levels of Vif had been reached. Consistent with data reported by Sheehy et al. (36), Vif did not affect APOBEC3G mRNA levels, which remained constant even at high concentrations of Vif (Fig. 5B, panel APOBEC3G, and C, RNA). These results suggest that Vif affects APOBEC3G at a posttranscriptional level.

Vif does not increase turnover but slows the rate of synthesis of APOBEC3G. To investigate the cause of the reduced APOBEC3G steady-state levels observed in the presence of Vif, pulse-chase experiments were performed to determine the half-life of cell-associated APOBEC3G in the presence or absence of Vif. Two sets of experiments were performed. For the first set (Fig. 6A to C), APOBEC3G was analyzed in the absence of virus production, while in the second set (Fig. 6D to F) APOBEC3G was assayed in virus-producing cells. HeLa cells were cotransfected with pcDNA-APO3G (2 μg) and either 3 μg of pNL-A1 (Fig. 6A to C) or 3 μg of pNL4-3 (Fig. 6D to F). Transfected cells were subjected to pulse-chase analysis as described in Materials and Methods. APOBEC3G-specific proteins were immunoprecipitated with a Myc epitope-specific polyclonal rabbit antibody and separated by 11% acrylamide-SDS gel electrophoresis followed by fluorography (Fig. 6A and D). APOBEC3G-specific bands were quantified by phosphorimaging analysis and plotted either as percentages of the input

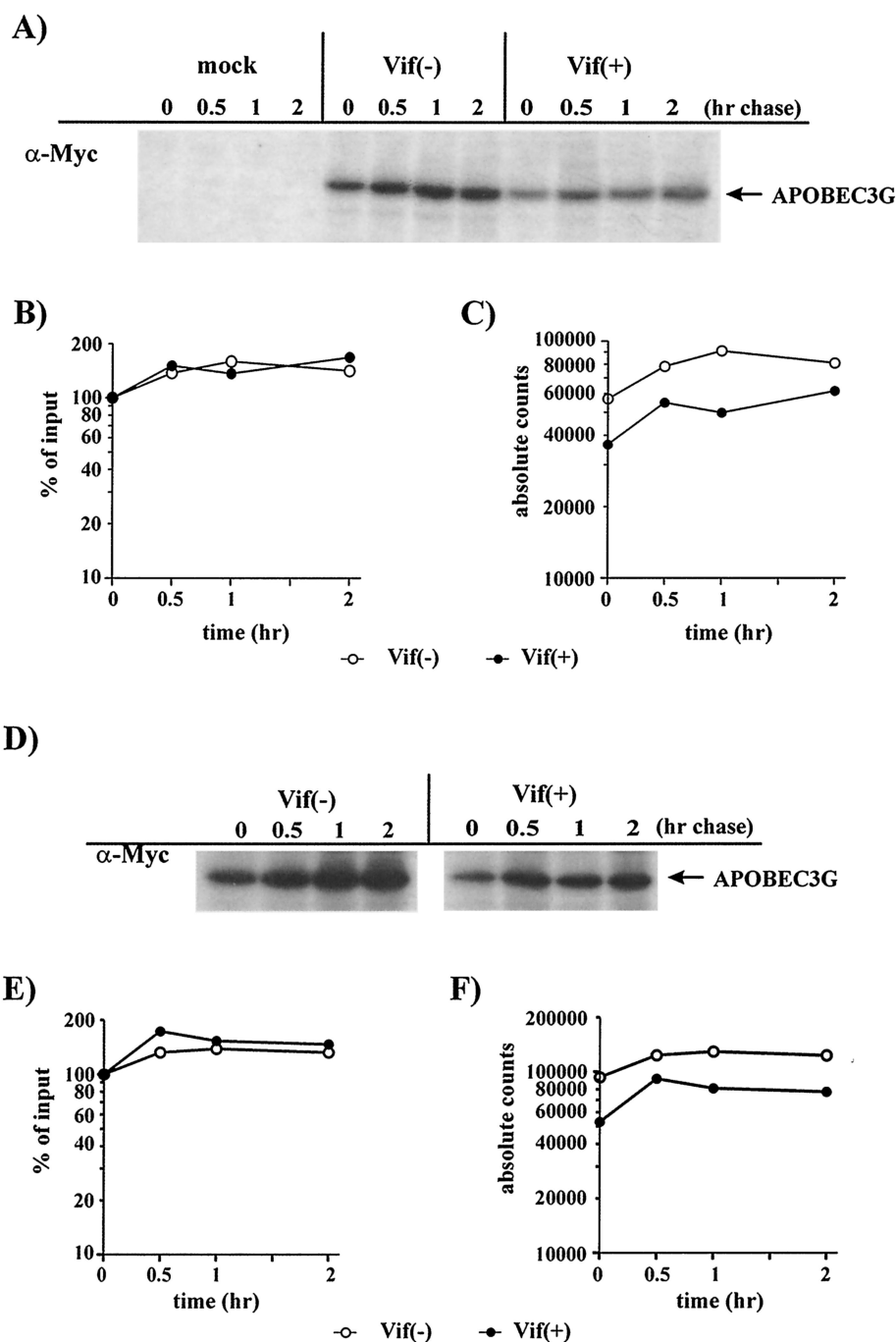


FIG. 6. Kinetic analysis of APOBEC3G. (A to C) Duplicate flasks of HeLa cells (25 cm²) were each transfected with 2 μ g of pcDNA3.1 vector and 3 μ g of pNL-A1 (mock), 2 μ g of pcDNA-APO3G and 3 μ g of pNL-A1vif(-) [Vif(-)], or 2 μ g of pcDNA-APO3G and 3 μ g of pNL-A1 [Vif(+)]. (D to F) Duplicate flasks of HeLa cells (25 cm²) were each transfected with 2 μ g of pcDNA-APO3G and 3 μ g of pNL4-3vif(-) [Vif(-)] or 2 μ g of pcDNA-APO3G and 3 μ g of wild-type pNL4-3 [Vif(+)]. Transfected cells were subjected to pulse-chase analysis followed by immunoprecipitation using a Myc-specific polyclonal rabbit antiserum as described in Materials and Methods. (A and D) Immunoprecipitated samples were subjected to 11% acrylamide-SDS gel electrophoresis and visualized by fluorometry. An increase in signal intensity was observed over time for all samples, presumably due to refolding of the protein. APOBEC3G-specific bands were quantified using a Fuji phosphorimager. Sample values were individually corrected for background and analyzed with Image Gauge, version 3.45, software (Fuji Photofilm LTD). In panels B and E, results are expressed as percentages of the starting sample (0 h of chase) and plotted as a function of time. In panels C and F, absolute phosphorimager values were plotted as a function of time.

values (time zero) (Fig. 6B and E) or as absolute phosphorimager values (Fig. 6C and F). Despite the fact that pNL-A1-transfected cells express about 10-fold higher levels of Vif than virus-producing pNL4-3-transfected cells, the results for both sets of experiments were virtually identical. In both experiments, Vif had no impact on the stability of APOBEC3G (Fig. 6B and E). Instead, the absolute amounts of immunoprecipitated APOBEC3G were reduced by about 30% in Vif-expressing cells throughout the time course of the experiment (Fig. 6C and F). These results are consistent with the 30% reduction in steady-state levels of APOBEC3G observed in Vif-expressing cells (Fig. 5) and suggest that Vif affects the rate of synthesis rather than the stability of APOBEC3G.

DISCUSSION

Replication of HIV-1 in primary target cells requires the activity of a number of accessory proteins whose specific functions have remained a mystery for many years. This includes the viral infectivity factor Vif, whose critical role for virus replication was recognized more than a decade ago (10, 44) but whose *modus operandi* has remained obscure. An important observation made early on was that Vif functions in a host cell-dependent manner. While this suggested that Vif engages in functional and/or physical interaction with one or more cellular factors, the identity of these factors remained unknown. A variety of Vif-interacting host factors have since been identified. These include vimentin (20), Hck (17), sp140 (28), and CEM15 (36). In fact, expression of Hck and CEM15 appeared to be associated with inhibition of viral infectivity in a Vif-dependent manner (17, 36). However, only CEM15 expression was closely linked to nonpermissive cellular phenotypes and, unlike Hck, did not seem to have additional effects on virus production. Thus, CEM15 represents to date the most promising factor that fits most, if not all, of the characteristics required of a protein associated with Vif-dependent host cell restriction: it is expressed exclusively in nonpermissive cells and expression in permissive cells inhibits virus infectivity in the absence but not in the presence of Vif (36).

Our data confirm the structural and functional identity of CEM15 and APOBEC3G and verify the inhibitory activity of this protein on HIV-1 replication when expressed in permissive HeLa cells. Our data further confirm the presence of APOBEC3G in virus particles and, in fact, suggest that packaging of APOBEC3G may contribute to its inhibitory activity. This conclusion is based on the results shown in Fig. 1 to 4, which reveal a clearer correlation of the APOBEC3G-imposed restriction with the levels of virus-associated protein than with the intracellular expression levels. In general, Vif expression resulted in a 20 to 30% reduction of cell-associated APOBEC3G compared to an up to 50-fold reduction in virus-associated protein. This suggests that Vif functions at several levels to reduce the intracellular levels and to inhibit packaging of APOBEC3G. Interestingly, reduction of the intracellular steady-state levels of APOBEC3G was observed for wild-type Vif but not for a series of biologically inactive Vif variants, adding further support to the notion that at least part of the activity of Vif involving APOBEC3G occurs intracellularly. Our results suggest that such Vif-induced reduction of cell-associated APOBEC3G is not due to an effect of Vif on the

expression or stability of APOBEC3G mRNA, and we did not find any evidence for an increased turnover of APOBEC3G in Vif-expressing HeLa cells. Instead, our kinetic analyses point to an effect of Vif on the rate of APOBEC3G protein synthesis. This effect of Vif was apparent irrespective of virus production.

How Vif interferes with the packaging of APOBEC3G remains subject to further investigation. However, since both Vif and APOBEC3G have RNA binding capability (9, 19, 21, 46), the most obvious mechanism by which Vif might interfere with APOBEC3G packaging—aside from reducing intracellular expression levels—is competitive binding to a common signal on the viral RNA. For such a mechanism to function efficiently, Vif would have to have a significantly higher binding affinity than APOBEC3G. Favoring such a model is the dose dependence of Vif-mediated inhibition of APOBEC3G packaging shown in Fig. 2 as well as the fact that, conversely, increasing levels of APOBEC3G did not affect Vif packaging. On the other hand, our identification of biologically inactive Vif mutants that are still efficiently packaged but have lost the ability to block packaging of APOBEC3G may argue against a competitive packaging process. Still, the requirements for Vif packaging are complex and may not only involve an interaction with the viral genomic RNA but, in addition, may entail binding to Gag precursor molecules (Akari and Strebel, unpublished data). The domains in Vif involved in RNA binding and interaction with Gag are likely distinct, and the presence of only one of these domains may be sufficient for Vif packaging; however, both domains in Vif may be required to inhibit packaging of APOBEC3G.

After submission of this work, a series of studies was submitted and published that provided convincing evidence that packaging of APOBEC3G into Vif-defective virions induces hypermutation of viral cDNA (15, 24, 29, 47). Two possible mechanisms were proposed to explain the effect of APOBEC3G-induced hypermutation on viral infectivity: (i) the increased mutation rate may affect viral fitness by introducing lethal mutations into the viral genome and (ii) alternatively, deamination of deoxycytidine to deoxyuridine could trigger an excision repair mechanism involving uracil DNA glycosylase (25) that could result in the degradation of viral cDNA prior to integration. The latter mechanism would be consistent with the observed inability of *vif*-defective viruses to produce full-length cDNAs in infected cells (40, 45). APOBEC3G did not cause hypermutation of the viral genomic RNA but specifically targeted minus-strand cDNA, implying that hypermutation is caused by virus-associated APOBEC3G. Thus, the ability of Vif to overcome the inhibitory effect of APOBEC3G may be directly related to its ability to inhibit packaging of the deaminase into viral particles. While our finding that Vif counteracts packaging of APOBEC3G is contradicted by a recent report by Harris et al. who maintain that physical transfer of APOBEC3G is not inhibited by Vif (15), our data are consistent with a more recent report by Mariani et al., submitted and published after submission of our article, demonstrating the species-specific exclusion of APOBEC3G from HIV-1 virions (30).

ACKNOWLEDGMENTS

We are grateful to Stephan Bour and Malcolm Martin for helpful discussions and for critically reading the manuscript. We thank Michael Malim for the Vif monoclonal antibody (MAb 319) and Jason

Roos and Janice Clements for the LuSIV indicator cell line. Both reagents were obtained through the NIH Research and Reference Reagent Program.

Part of this work was supported by a grant from the NIH Intramural AIDS Targeted Antiviral Program to K.S.

REFERENCES

- Adachi, A., H. E. Gendelman, S. Koenig, T. Folks, R. Willey, A. Rabson, and M. A. Martin. 1986. Production of acquired immunodeficiency syndrome-associated retrovirus in human and nonhuman cells transfected with an infectious molecular clone. *J. Virol.* **59**:284–291.
- Akari, H., T. Uchiyama, T. Fukumori, S. Iida, A. H. Koyama, and A. Adachi. 1999. Pseudotyping human immunodeficiency virus type 1 by vesicular stomatitis virus G protein does not reduce the cell-dependent requirement of vif for optimal infectivity: functional difference between Vif and Nef. *J. Gen. Virol.* **80**:2945–2949.
- Bennett, M. K., N. Calakos, and R. H. Scheller. 1992. Syntaxin: a synaptic protein implicated in docking of synaptic vesicles at presynaptic active zones. *Science* **257**:255–259.
- Borman, A. M., C. Quillent, P. Charneau, C. Dauguet, and F. Clavel. 1995. Human immunodeficiency virus type 1 Vif-mutant particles from restrictive cells: role of Vif in correct particle assembly and infectivity. *J. Virol.* **69**:2058–2067.
- Bour, S., and K. Strebel. 2000. HIV accessory proteins: multifunctional components of a complex system. *Adv. Pharmacol.* **48**:75–120.
- Bouyac, M., F. Rey, M. Nascimbeni, M. Courcoul, J. Sire, D. Blanc, F. Clavel, R. Vigne, and B. Spire. 1997. Phenotypically Vif[−] human immunodeficiency virus type 1 is produced by chronically infected restrictive cells. *J. Virol.* **71**:2473–2477.
- Chowdhury, I. H., W. Chao, M. J. Potash, P. Sova, H. E. Gendelman, and D. J. Volsky. 1996. vif-negative human immunodeficiency virus type 1 persistently replicates in primary macrophages, producing attenuated progeny virus. *J. Virol.* **70**:5336–5345.
- Courcoul, M., C. Patience, F. Rey, D. Blanc, A. Harmache, J. Sire, R. Vigne, and B. Spire. 1995. Peripheral blood mononuclear cells produce normal amounts of defective Vif[−] human immunodeficiency virus type 1 particles which are restricted for the preretrotranscription steps. *J. Virol.* **69**:2068–2074.
- Dettenhofer, M., S. Cen, B. A. Carlson, L. Kleiman, and X. F. Yu. 2000. Association of human immunodeficiency virus type 1 Vif with RNA and its role in reverse transcription. *J. Virol.* **74**:8938–8945.
- Fisher, A. G., B. Ensoli, L. Ivanoff, M. Chamberlain, S. Petteway, L. Ratner, R. C. Gallo, and F. Wong-Staal. 1987. The *src* gene of HIV-1 is required for efficient virus transmission in vitro. *Science* **237**:888–893.
- Fouchier, R. A., B. E. Meyer, J. H. Simon, U. Fischer, and M. H. Malim. 1997. HIV-1 infection of non-dividing cells: evidence that the amino-terminal basic region of the viral matrix protein is important for Gag processing but not for post-entry nuclear import. *EMBO J.* **16**:4531–4539.
- Fouchier, R. A., J. H. Simon, A. B. Jaffe, and M. H. Malim. 1996. Human immunodeficiency virus type 1 Vif does not influence expression or virion incorporation of *gag*-, *pol*-, and *env*-encoded proteins. *J. Virol.* **70**:8263–8269.
- Fujita, M., A. Sakurai, A. Yoshida, S. Matsumoto, M. Miyaura, and A. Adachi. 2002. Subtle mutations in the cysteine region of HIV-1 Vif drastically alter the viral replication phenotype. *Microbes Infect.* **4**:621–624.
- Gaddis, N. C., E. Chertova, A. M. Sheehy, L. E. Henderson, and M. H. Malim. 2003. Comprehensive investigation of the molecular defect in vif-deficient human immunodeficiency virus type 1 virions. *J. Virol.* **77**:5810–5820.
- Harris, R. S., K. N. Bishop, A. M. Sheehy, H. M. Craig, S. K. Petersen-Mahrt, I. N. Watt, M. S. Neuberger, and M. H. Malim. 2003. DNA deamination mediates innate immunity to retroviral infection. *Cell* **113**:803–809.
- Harris, R. S., S. K. Petersen-Mahrt, and M. S. Neuberger. 2002. RNA editing enzyme APOBEC1 and some of its homologs can act as DNA mutators. *Mol. Cell* **10**:1247–1253.
- Hassaine, G., M. Courcoul, G. Bessou, Y. Barthalay, C. Picard, D. Olive, Y. Collette, R. Vigne, and E. Decroly. 2001. The tyrosine kinase Hck is an inhibitor of HIV-1 replication counteracted by the viral Vif protein. *J. Biol. Chem.* **276**:16885–16893.
- Hoglund, S., A. Ohagen, K. Lawrence, and D. Gabuzda. 1994. Role of vif during packing of the core of HIV-1. *Virology* **201**:349–355.
- Jarmuz, A., A. Chester, J. Bayliss, J. Gisbourne, I. Dunham, J. Scott, and N. Navaratnam. 2002. An anthropoid-specific locus of orphan C to U RNA-editing enzymes on chromosome 22. *Genomics* **79**:285–296.
- Karczewski, M. K., and K. Strebel. 1996. Cytoskeleton association and virion incorporation of the human immunodeficiency virus type 1 Vif protein. *J. Virol.* **70**:494–507.
- Khan, M. A., C. Aberham, S. Kao, H. Akari, R. Gorelick, S. Bour, and K. Strebel. 2001. Human immunodeficiency virus type 1 Vif protein is packaged into the nucleoprotein complex through an interaction with viral genomic RNA. *J. Virol.* **75**:7252–7265.
- Khan, M. A., H. Akari, S. Kao, C. Aberham, D. Davis, A. Buckler-White, and K. Strebel. 2002. Intravirion processing of human immunodeficiency virus type 1 Vif protein by the viral protease may be correlated with Vif function. *J. Virol.* **76**:9112–9123.
- Kishi, M., Y. Nishino, M. Sumiya, K. Ohki, T. Kimura, T. Goto, M. Nakai, M. Kakinuma, and K. Ikuta. 1992. Cells surviving infection by human immunodeficiency virus type 1: vif or vpu mutants produce non-infectious or markedly less cytopathic viruses. *J. Gen. Virol.* **73**:77–87.
- Lecossier, D., F. Bouchonnet, F. Clavel, and A. J. Hance. 2003. Hypermutation of HIV-1 DNA in the absence of the Vif protein. *Science* **300**:1112.
- Lindahl, T., and R. D. Wood. 1999. Quality control by DNA repair. *Science* **286**:1897–1905.
- Ma, X. Y., P. Sova, W. Chao, and D. J. Volsky. 1994. Cysteine residues in the Vif protein of human immunodeficiency virus type 1 are essential for viral infectivity. *J. Virol.* **68**:1714–1720.
- Madani, N., and D. Kabat. 1998. An endogenous inhibitor of human immunodeficiency virus in human lymphocytes is overcome by the viral Vif protein. *J. Virol.* **72**:10251–10255.
- Madani, N., R. Millette, E. J. Platt, M. Marin, S. L. Kozak, D. B. Bloch, and D. Kabat. 2002. Implication of the lymphocyte-specific nuclear body protein sp140 in an innate response to human immunodeficiency virus type 1. *J. Virol.* **76**:11133–11138.
- Mangeat, B., P. Turelli, G. Caron, M. Friedli, L. Perrin, and D. Trono. 2003. Broad antiretroviral defence by human APOBEC3G through lethal editing of nascent reverse transcripts. *Nature* **424**:99–103.
- Mariani, R., D. Chen, B. Schrofelbauer, F. Navarro, R. Konig, B. Bollman, C. Munk, H. Nymark-McMahon, and N. R. Landau. 2003. Species-specific exclusion of APOBEC3G from HIV-1 virions by Vif. *Cell* **114**:21–31.
- Ochsenbauer, C., T. Wilk, and V. Bosch. 1997. Analysis of vif-defective human immunodeficiency virus type 1 (HIV-1) virions synthesized in “non-permissive” T lymphoid cells stably infected with selectable HIV-1. *J. Gen. Virol.* **78**:627–635.
- Ohagen, A., and D. Gabuzda. 2000. Role of vif in stability of the human immunodeficiency virus type 1 core. *J. Virol.* **74**:11055–11066.
- Reddy, T. R., G. Kraus, O. Yamada, D. J. Looney, M. Suhasini, and F. Wong-Staal. 1995. Comparative analyses of human immunodeficiency virus type 1 (HIV-1) and HIV-2 Vif mutants. *J. Virol.* **69**:3549–3553.
- Roos, J. W., M. F. Maughan, Z. Liao, J. E. Hildreth, and J. E. Clements. 2000. LuSIV cells: a reporter cell line for the detection and quantitation of a single cycle of HIV and SIV replication. *Virology* **273**:307–315.
- Sakai, K., M. Horiuchi, S. Iida, T. Fukumori, H. Akari, and A. Adachi. 1999. Mutational analysis of human immunodeficiency virus type 1 vif gene. *Virus Genes* **18**:179–181.
- Sheehy, A. M., N. C. Gaddis, J. D. Choi, and M. H. Malim. 2002. Isolation of a human gene that inhibits HIV-1 infection and is suppressed by the viral Vif protein. *Nature* **418**:646–650.
- Simm, M., M. Shahabuddin, W. Chao, J. S. Allan, and D. J. Volsky. 1995. Aberrant Gag protein composition of a human immunodeficiency virus type 1 vif mutant produced in primary lymphocytes. *J. Virol.* **69**:4582–4586.
- Simon, J. H., R. A. Fouchier, T. E. Southerling, C. B. Guerra, C. K. Grant, and M. H. Malim. 1997. The Vif and Gag proteins of human immunodeficiency virus type 1 colocalize in infected human T cells. *J. Virol.* **71**:5259–5267.
- Simon, J. H., N. C. Gaddis, R. A. Fouchier, and M. H. Malim. 1998. Evidence for a newly discovered cellular anti-HIV-1 phenotype. *Nat. Med.* **4**:1397–1400.
- Simon, J. H., and M. H. Malim. 1996. The human immunodeficiency virus type 1 Vif protein modulates the postpenetration stability of viral nucleoprotein complexes. *J. Virol.* **70**:5297–5305.
- Simon, J. H., A. M. Sheehy, E. A. Carpenter, R. A. Fouchier, and M. H. Malim. 1999. Mutational analysis of the human immunodeficiency virus type 1 Vif protein. *J. Virol.* **73**:2675–2681.
- Simon, J. H., T. E. Southerling, J. C. Peterson, B. E. Meyer, and M. H. Malim. 1995. Complementation of vif-defective human immunodeficiency virus type 1 by primate, but not nonprimate, lentivirus vif genes. *J. Virol.* **69**:4166–4172.
- Steffens, C. M., and T. J. Hope. 2001. Recent advances in the understanding of HIV accessory protein function. *AIDS* **15**(Suppl. 5):S21–S26.
- Strebel, K., D. Daugherty, K. Clouse, D. Cohen, T. Folks, and M. A. Martin. 1987. The HIV “A” (src) gene product is essential for virus infectivity. *Nature* **328**:728–730.
- von Schwedler, U., J. Song, C. Aiken, and D. Trono. 1993. Vif is crucial for human immunodeficiency virus type 1 proviral DNA synthesis in infected cells. *J. Virol.* **67**:4945–4955.
- Zhang, H., R. J. Pomerantz, G. Dornadula, and Y. Sun. 2000. Human immunodeficiency virus type 1 Vif protein is an integral component of an mRNP complex of viral RNA and could be involved in the viral RNA folding and packaging process. *J. Virol.* **74**:8252–8261.
- Zhang, H., B. Yang, R. J. Pomerantz, C. Zhang, S. C. Arunachalam, and L. Gao. 2003. The cytidine deaminase CEM15 induces hypermutation in newly synthesized HIV-1 DNA. *Nature* **424**:94–98.